Claims 7-12 are pending. Claims 1-6 were previously cancelled as drawn to a nonelected election in view of the Restriction Requirement. Claim 7 has been amended to increase the clarity of the claimed invention. In particular, in the second line of claim 7, two typographical errors have been corrected. Support for the corrections is found in claim 7 as originally filed. Further, language has been added to (b) of claim 7 to clarify the claimed DNA sequences that hybridize. The DNA sequences encode amino acid sequences that are native to HER-2/*neu* protein and that are approximately the same length as the polypeptide encoded by the DNA sequence of (a) of claim 7. Support for the language is found, in part, at page 3, lines 18-19, and page 30, lines 10-24, of the subject specification. No new matter has been added. The term "approximately" carries its usual meaning of within five percent. Therefore, amended claims 7-12 are now pending.

As a preliminary matter, it is respectfully requested that the Examiner provide an initialed copy of the Form PTO-1449 submitted by Applicants on October 13, 1999.

In the Office Action dated November 22, 2000, claims 7-12 were rejected under 35 U.S.C. § 112, first paragraph. This rejection is respectfully traversed.

The rejection is divided into two parts, (A) and (B), which correspond to DNA sequences of (a) and (b), respectively, of claim 7. Rejection part (A), as set forth at pages 2-4 of the Office Action, is addressed first. In summary, the specification is asserted to not be enabling on the grounds that, in the absence of data, one cannot apply the teachings of the specification to the claims due to the highly unpredictable nature of administration of nucleic acids into a host. In support of this assertion, three journal articles are cited at pages 3-4 of the Office Action: Davis et al. (Human Gene Therapy 4:151-159, 1993); Wickham (Gene Therapy 7:110-114, 2000); and Crystal (Cancer Chemother. Pharmacol. 43:590-599, 1999).

Applicants respectfully, but strenuously disagree. As a starting point, Applicants wish to make clear that their invention is concerned with immunization with DNA, and not with gene therapy. Immunization with DNA and gene therapy are separate and distinct concepts. Immunization with DNA is to generate an immune response to the expressed protein encoded by the DNA. Gene therapy is to produce the protein encoded by the DNA so that the protein will function where its activity is needed because it is absent or underexpressed. Unlike immunization with DNA, for gene therapy an immune response to the expressed protein is not

desired because it will result in inactivation of the protein. For gene therapy it is usually important to achieve a permanent level of expression of the nominated protein. For immunization it is not important to achieve a permanent level of expression. A major problem with gene therapy is variable expression as pointed out in Davis et al (Human Gene Therapy 4: 151-159, 1993). Another major problem with gene therapy is that expression is often short lived. Immunization, however, requires only transient expression. The point of immunization is to achieve short-term expression of the nominated protein. The purpose is to elicit an immune response to the expressed protein. Once the immune response is generated, cells expressing the protein can be destroyed by the immune response. That is the point of immunization – to induce an immune response capable of killing the cells that express the protein. Often then the expression vector is rejected. Rejection of the vector that expresses the protein prevents successful gene therapy. Rejection of the vector that expressed the protein is considered to be the goal of immunization and is considered to be a positive outcome. Once an immune response has been generated it is no longer necessary that the cell containing the plasmid continue to express protein. The difference between gene therapy and immunization was well pointed out in a 1995 review article by Wilkinson and Borysiewicz (Br. Med Bull; abstract provided herewith). They state, "A similar vector technology directed at in vivo gene delivery is currently being exploited both for gene therapy and vaccination. The induction of an immune response to an expressed transgene represents a potential hazard for a gene therapy protocol but is the object of a vaccine strategy. In vivo gene delivery using replication-competent or replication-deficient viral vector systems and by direct transfer of naked DNA can generate an effective humeral, secretory and cell-mediated immune response to expressed transgenes."

Davis et al. is cited in the Office Action for the proposition that following injection of DNA into striated muscle, the fibers damaged by the injection procedure do not take up DNA and high levels of expression can damage muscle cells. However, expression of DNA in muscle is not necessary for immunization with DNA. The article by Davis et al. speaks to issues important for gene therapy, i.e., the ability or transferred genes to express the nominated protein in striated muscle. Immunization with genes is an entirely different concept.

Furthermore, for DNA immunization regimens in which DNA is injected into striated muscle, DNA is actually expressed in dendritic cells in vivo, irrespective of where the DNA is injected. The immune response is elicited by T cell recognition of protein expressed in dendritic cells, not by protein expressed in striated muscle. Of note is the 1996 article by Corr et al. (J. Exp. Med.; abstract provided herewith) showing the immunization by injection of DNA into muscle results in eventual expression of DNA in bone marrow derived cells in vivo (presumably dendritic cells). It is the expression of DNA in bone marrow derived antigen presenting cells that is most important, not expression in muscle cells. The concept is supported by the 1996 article of Condon et al. (Nat. Med.; abstract provided herewith) showing that immunization with DNA cutaneously results in expression of the nominate protein in skin-derived dendritic cells. Thus, although targeting of genes to particular organs may be essential for gene therapy, it is not essential for immunization with genes.

The specification is alleged to lack enablement for providing no direction as to tissue specific targeting or tumor specific targeting. In this regard, the article by Wickham is referenced. Whereas specific targeting may be essential for gene therapy, specific targeting is not essential for immunization. As presented above, immunization with DNA intramuscularly or cutaneously and presumably subcutaneously, the most common routes for immunization, result in essential expression in bone marrow derived dendritic cells and not the injected tissue. For gene therapy regimens designed to express a protein lacking in tumors, it is essential that the protein be expressed in the tumor. For immunization, there is no need for expression in tumor. Immunization elicits an immune response. The T cells which are elicited travel to sites of tumor.

The specification is alleged to lack enablement for providing no direction as to the replication competence of the viral vector for the delivery of the polynucleotide and the persistence time of the virus. The article by Crystal (Cancer Chemother. Pharmacol. 43:590-599, 1999), reviews the use of viral vectors to express antigens in dendritic cells and the subsequent use of the infected dendritic cells for immunization. There is not a mandatory need for defective viruses. Natural occurring viruses are exceedingly immunogenic. Although as Crystal points out that defective viruses might be helpful for ex vivo approaches, such viruses have in no way been proven to be mandatory for direct immunization approaches. Further, if replication incompetent

virus is desired, the methodology was available in the art at the time the present application was filed (e.g., art cited at pages 32-33).

Therefore, contrary to the assertion in part (A) of the rejection, the administration of nucleic acids into a host for immunization purposes is not highly unpredictable once one has discovered that the protein expressed by the nucleic acids will elicit a T cell response.

Nevertheless, in order to expedite prosecution, Applicants submit herewith a Declaration pursuant to 37 C.F.R. § 1.132 which confirms that a viral vector expressing the intracellular domain (ICD) of HER/2-*neu* generates ICD-specific T cell activity. Further confirmation is provided in an article submitted herewith wherein mice immunized with ICD plasmid DNA were protected against growth of a HER-2/*neu*-expressing murine tumor.

Rejection part (B) is set forth at pages 4-5 of the Office Action. In summary, the specification is asserted to not be enabling on the grounds that small fragments and non-natural variants of the polypeptide encoded by (a) of claim 7 are not taught, but could hybridize according to (b) of claim 7.

Applicants respectfully, but strenuously, disagree. Small fragments of the polypeptide encoded by (a) of claim 7 have been taught in Applicants' prior related applications. For example, U.S. Serial No. 414,417 (filed March 31, 1995) is listed at page 1, line 9, of the subject application as filed and is incorporated by reference at page 47, lines 28-32. That prior application matured into U.S. Patent No. 5,801,005 in which columns 11-12 contain peptides (i.e., small fragments) of the polypeptide encoded by (a) of claim 7. Variants of the polypeptide are described in the subject application as filed at pages 12-17. This disclosure includes at pages 13-14 the citation to useful computer programs for epitope identification. Thus, Applicants respectfully disagree that small fragments and non-natural variants of the polypeptide encoded by (a) of claim 7 have not been taught in the subject application.

Nevertheless, in order to expedite prosecution, claim 7 has been further amended to clarify that the claimed DNA sequences that would hybridize according to (b) of claim 7 encode polypeptides that are native to HER-2/*neu* protein (i.e., naturally occurring) and are approximately the same length as the polypeptide encoded by (a) of claim 7. Accordingly, without conceding the issue of the patentability of claim 7 as originally filed, Applicants believe

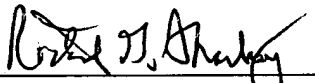
that (b) of amended claim 7 (and thus claims 8-12 which depend from claim 7) is commensurate in scope with the teachings of the subject specification.

Therefore, Applicants believe that the rejection of claims 7-12 under 35 U.S.C. § 112, first paragraph, has been overcome. Withdrawal of this rejection is respectfully requested.

Therefore, in light of the amendments and remarks set forth above, Applicants believe that all the Examiner's rejections have been overcome. Reconsideration of this application and allowance of the pending claims (7-12) are respectfully requested. If there is any further matter requiring attention prior to allowance of the subject application, the Examiner is respectfully requested to contact the undersigned attorney (at 206-622-4900) to resolve the matter.

Respectfully submitted,

Seed Intellectual Property Law Group PLLC



Richard G. Sharkey, Ph.D.
Registration No. 32,629

Enclosures:

- Postcard
- Check
- Form PTO/SB/21
- Form PTO/SB/17 (+ copy)
- Petition for an Extension of Time (+copy)
- Abstracts (3)
- Journal Article
- 1.132 Declaration

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

At page 1, before the Technical Field, the Cross Reference to Related Applications has been amended as follows:

This application is a continuation of U.S. Patent Application No. 08/625,101, filed ~~April 1, 1996, which issued as U.S. Patent No. 5,869,445;~~ which application is a continuation-in-part ~~continuation~~ of U.S. Patent Application No. 08/414,417, filed March 31, 1995, which issued as U.S. Patent No. 5,801,005; which application is a continuation-in-part of U.S. Patent Application No. 08/106,112, filed on August 12, 1993, abandoned; which application is a continuation-in-part of U.S. Patent Application No. 08/033,644, filed March 17, 1993, abandoned.

In the Claims:

Claim 7 has been amended as follows:

7. (Twice Amended) A method for eliciting or enhancing an immune response to HER/2-*neu* protein, comprising administering to a warm-blooded ~~warm blooded~~ animal in an ~~a~~ amount effective to elicit or enhance said response a nucleic acid molecule or a viral vector wherein the nucleic acid molecule or the viral vector directs the expression of a polypeptide encoded by a DNA sequence selected from:

(a) nucleotides 2026 through 3765 of SEQ ID NO:1; and

(b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 2026 through 3765 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that produces an immune response to HER-2/*neu* protein and that possesses an amino acid sequence which is identical to a portion of HER-2/*neu* protein and which is approximately the same length as the polypeptide encoded by the DNA sequence of (a).

PMID: 11249767

Br Med Bull 1995 Jan;51(1):205-16

Gene therapy and viral vaccination: the interface.

Wilkinson GW, Borysiewicz LK.

Department of Medicine, University of Wales College of Medicine, Cardiff, UK.

Live viral vaccines have had a major impact on the incidence of acute virus infections world-wide. Virus infections recognised as future vaccine targets will require a modified approach based on a detailed understanding of the immunobiology of specific infections combined with the application of new technologies designed to generate specific and appropriate protective immunity. A similar vector technology directed at in vivo gene delivery is currently being exploited both for gene therapy and vaccination. The induction of an immune response to an expressed transgene represents a potential hazard for a gene therapy protocol but is the object of a vaccine strategy. In vivo gene delivery using replication-competent or replication-deficient viral vector systems and by direct transfer of naked DNA can generate an effective humoral, secretory and cell-mediated immune response to expressed transgenes.

PMID: 7767644

J Exp Med 1996 Oct 1;184(4):1555-60

Gene vaccination with naked plasmid DNA: mechanism of CTL priming.

Corr M, Lee DJ, Carson DA, Tighe H.

Department of Medicine, University of California, San Diego, La Jolla 92093-0663, USA.

The injection of naked plasmid DNA directly into the muscle cells of mice has been shown to induce potent humoral and cellular immune responses. The generation of a cytotoxic T lymphocyte (CTL) response after plasmid DNA injection may involve the presentation of the expressed antigen in the context of the injected myocytes' endogenous major histocompatibility (MHC)-encoded class I molecules or may use the MHC molecules of bone marrow-derived antigen presenting cells (APC) which are capable of providing co-stimulation as well. To resolve which cell type provides the specific restricting element for this method of vaccination we generated parent-->F1 bone marrow chimeras in which H-2bxd recipient mice received bone marrow that expressed only H-2b or H-2d MHC molecules. These mice were injected intramuscularly with naked plasmid DNA that encoded the nucleoprotein from the A/PR/8/34 influenza strain, which as a single antigen has epitopes for both H-2Db and H-2Kd. The resulting CTL responses were restricted to the MHC haplotype of the bone marrow alone and not to the second haplotype expressed by the recipient's myocytes. The role of somatic tissues that express protein from injected plasmids may be to serve as a reservoir for that antigen which is then transferred to the APC. Consequently, our data show that the mechanism of priming in this novel method for vaccination uses the MHC from bone marrow-derived APC, which are efficient at providing all of the necessary signals for priming the T cell.

Nat Med 1996 Oct; 2(10):1122-8

DNA-based immunization by in vivo transfection of dendritic cells.

Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr.

Department of Dermatology, University of Pittsburgh School of Medicine, Pennsylvania 15213, USA.

Delivery of antigen in a manner that induces effective, antigen-specific immunity is a critical challenge in vaccine design. Optimal antigen presentation is mediated by professional antigen-presenting cells (APCs) capable of taking up, processing and presenting antigen to T cells in the context of costimulatory signals required for T-cell activation. Developing immunization strategies to optimize antigen presentation by dendritic cells, the most potent APCs, is a rational approach to vaccine design. Here we show that cutaneous genetic immunization with naked DNA results in potent, antigen-specific, cytotoxic T lymphocyte-mediated protective tumor immunity. This method of immunization results in the transfection of skin-derived dendritic cells, which localize in the draining lymph nodes. These observations provide a basis for further development of DNA-based vaccines and demonstrate the feasibility of genetically engineering dendritic cells in vivo.

ELSEVIER

Vaccine 19 (2001) 2598–2606

Vaccine

www.elsevier.com/locate/vaccine

Vaccination with Her-2/neu DNA or protein subunits protects against growth of a Her-2/neu-expressing murine tumor

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Corixa Corporation, 1124 Columbia St. Suite 200, Seattle, WA 98104, USA

Abstract

The present study utilizes an in vivo murine tumor expressing human Her-2/neu to evaluate potential Her-2/neu vaccines consisting of either full length or various subunits of Her-2/neu delivered in either protein or plasmid DNA form. Our results demonstrate that protective immunity against Her-2/neu-expressing tumor challenge can be achieved by vaccination with plasmid DNA encoding either full length or subunits of Her-2/neu. Partial protective immunity was also observed following vaccination with the intracellular domain (ICD), but not extracellular domain (ECD), protein subunit of Her-2/neu. The mechanism of protection elicited by plasmid DNA vaccination appeared to be exclusively CD4 dependent, whereas the protection observed with ICD protein vaccination required both CD4 and CD8 T cells.

1. Introduction

Her-2/neu is a proto-oncogene encoding a 185 kD protein with homology to the epidermal growth factor receptor (EGFR) family [1,2]. It is over-expressed in several human cancers including breast, ovarian, and gastric carcinomas [3,4]. Studies of patients with Her-2/neu expressing tumors have demonstrated the existence of *anti*-Her-2/neu antibody and T cell responses [5–8], yet in most cases, the immune responses in these patients is insufficient to provide protection against tumor growth. Other groups have demonstrated that Her-2/neu-specific cytotoxic T cells (CTL) or T helper cells (Th) can be generated through in vitro stimulation of T cells with autologous Her-2/neu-expressing tumor cells or Her2-neu derived peptides, indicating that in some patients the potential exists for boosting *anti*-Her-2/neu immunity [5,7,9–11]. Taken together, these studies provide support for Her-2/neu as a candidate for a tumor antigen vaccine.

The only current approved Her-2/neu immunotherapy is passive transfer of a Her-2/neu monoclonal antibody (reviewed in [12]). Although this has been shown to be effective at inhibiting tumor growth in a limited population of Her-2/neu patients, it is possible that elicitation of an active and more comprehensive immune response that includes both antibody and T cell responses may provide more effective anti-tumor immunity. The present study utilizes an in vivo murine tumor expressing human Her-2/neu to evaluate several potential Her-2/neu vaccines. In this study we have evaluated vaccines consisting of either full length or various subunits of Her-2/neu delivered in either protein or plasmid DNA form. Our results demonstrate that protective immunity against Her-2/neu-expressing tumor challenge can be achieved by vaccination with plasmid DNA encoding either full length or subunits of Her-2/neu. Partial protective immunity was also observed following vaccination with the intracellular domain (ICD), but not extracellular domain (ECD), protein subunit of Her-2/neu. Interestingly, the mechanism of protection elicited by plasmid DNA vaccination appeared to be exclusively CD4 dependent, whereas the protection observed with ICD protein vaccination required both CD4 and CD8 T cells.

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0264-410X/01/$ - see front matter
PII: S0264-410X(00)00493-X

2. Materials and methods

2.1. Animals

Eight to 12 week old female C57Bl/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in our animal facility at Corixa Corporation.

2.2. Antibodies and reagents

Rat *anti*-murine CD4 (GK1.5) and rat *anti*-murine CD8 (2.43) hybridoma cell lines were obtained from ATCC. Antibody was purified from ascites fluid. Ab-5, an *anti*-human Her-2/neu ECD-specific antibody, was purchased from Oncogene Research Products (Cambridge, MA). Montanide 720 was purchased from Seppic Inc. (Fairfield, NJ).

2.3. Tumor cell lines

EL4, a murine thymoma originally derived from C57BL mice, was obtained from ATCC. EL4 cells were transfected with full length human Her-2/neu using a standard electroporation protocol. EL4 cells stabely expressing Her-2/neu were obtained following in vitro drug selection with neomycin. Her-2/neu expression was confirmed by flow cytometric analysis.

2.4. Her2/neu vaccines

Her-2/neu plasmid DNA vaccine (pVR1012-Her-2/neu) consisted of the full length human Her-2/neu cDNA inserted into VR1012 (Vical, San Diego, CA). The ECD plasmid DNA vaccine (pVR1012-ECD) consisted of DNA encoding amino acids 1–695 of Her-2/neu and the ICD plasmid DNA vaccine (pVR1012-ICD) consisted of DNA encoding amino acids 692–1256 in VR1012. Large quantities of endotoxin free plasmid DNA were prepared using Qiagen Inc. (Valencia, CA) kit reagents and standard techniques. Plasmid DNA vaccines were delivered intramuscularly (100 ug) on day 0 and 21. ICD (amino acids 676–1256) and ECD (amino acids 22–653) recombinant subunit proteins were produced at Corixa Corporation. Briefly, ECD protein was produced by stable transfection of L cells and purified using a combination of DEAE, reverse phase HPLC, and Mono S column chromatography. ICD protein was produced in *E. coli* and purified from solubilized inclusion bodies via High Q anion exchange, followed by nickel resin affinity chromatography. Recombinant protein vaccines were mixed with Montanide 720 at a 7:2 (Montanide 720:protein) ratio and delivered subcutaneously.

2.5. In vivo tumor model

To ensure a consistent source of EL4-Her-2/neu cells for tumor protection experiments, cells were expanded by in vivo passage (i.p.) and frozen in aliquots for use in individual experiments. Tumors were established using 200 000 EL4-Her-2/neu cells injected subcutaneously on the flank. Palpable tumors typically developed within 8–10 days of injection. Tumor size is expressed in mm^2 as determined by measuring the area (length × width) of the tumors with a microcaliper device.

2.6. In vivo depletion of effector T cells

Mice were immunized with plasmid DNA or protein on days 0 and 21 to generate effector T cells. CD4 and CD8 cells were depleted by i.p. administration of 100 ug per day of purified *anti*-CD4 or *anti*-CD8 antibody on day 35, 38, and 42 following initiation of the experiment. Flow cytometric analysis of depleted splenocytes indicated greater than 98% depletion of the target populations.

2.7. Adoptive transfer of immune sera

Immune sera were obtained through bleeds of Her-2/neu plasmid DNA- or ICD protein-immunized mice. Sera from 12 individual mice from each group were pooled for transfer (i.v.) into six naïve recipient mice. Anti-Her-2/neu antibody titers of immune sera were assessed by ELISA prior to sera transfer.

2.8. In vitro cytokine analysis

Mice (four per group) were immunized with 100 ug pVR1012 or pVR1012-Her-2/neu (i.m) or 50 ug of ICD protein in Montanide (s.q.), or Montanide alone on day 0 and 21. Two weeks following the second immunization, 250 000 spleen cells were harvested and stimulated in vitro with media alone, ICD or ECD protein (10 μg/ml). IFNγ secretion was assayed by ELISA from supernatants taken 48 h following in vitro stimulation. Values represent the mean of tripicate wells for four individual mice.

3. Results

3.1. Her-2/neu protein subunit and plasmid DNA vaccines mediate tumor protection

Her 2/neu vaccines consisting of either full length or truncated forms of Her-2/neu were evaluated for the ability to elicit a protective immune response against challenge with a syngeneic Her-2/neu expressing tumor

cell line. C57Bl/6 mice were immunized with plasmid DNA encoding full length human Her-2/neu, ICD, or the ECD portions of Her-2/neu. Following two DNA immunizations, mice were challenged subcutaneously with EL4 cells transfected with full length human Her-2/neu (EL4-Her-2/neu) and tumor growth was monitored. In naïve mice, EL4-Her-2/neu cells form large solid tumors within 14–20 days of subcutaneous administration. Vaccination with Her-2/neu plasmid DNA, either full length, ICD or ECD subunits, substantially inhibits the growth of the tumor cells (Fig. 1). The majority of mice are completely protected from developing tumor, whereas as small portion of animals demonstrate a delay in tumor development for up to 3 weeks following tumor challenge. It is interesting to note that similar levels of tumor protection are achieved with both the truncated and the full length Her-2/neu constructs.

To determine whether protein subunit vaccines were also effective at eliciting tumor protection, mice were immunized with ICD or ECD protein plus adjuvant, challenged with EL4-Her-2/neu, and monitored for tumor growth. The results, shown in Fig. 2, demonstrate that vaccination with ICD protein elicits a partially protective immune response in which both the frequency of mice developing tumor and the mean tumor size of mice bearing tumors are decreased. In this representative experiment, ICD vaccination results in complete protection of one animal, and a decrease in mean tumor size of the mice developing tumors (162 mm^2on d23). This is compared to tumor growth in 4/4 mice (mean tumor size of 527 mm^2) in the naïve group and 4/4 mice (mean tumor size of 462 mm^2) in the ECD vaccinated group. Although the protection observed with ICD protein is only partial, we consistently observed a decrease in tumor size or complete protection in the majority of mice vaccinated with ICD protein. In comparison to Her-2/neu DNA vaccination however, it is clear that with the formulation and regimen employed protein vaccination is not as efficacious as plasmid DNA.

In order to determine whether the protection observed in this model was Her-2/neu specific, mice were vaccinated with full length Her-2/neu, or vector control plasmid DNA, and subsequently challenged with either parental EL4 or EL4-Her-2/neu cells. Growth of the tumors was monitored over the next 10–25 days. The results, depicted in Fig. 3, demonstrate that prevention of tumor growth only occurs in mice immunized with Her-2/neu plasmid DNA, suggesting that immunity to Her-2/neu is elicited and required for protection. Further evidence that tumor protection is Her-2/neu specific is provided by the observation that vaccination with Her-2/neu plasmid DNA does not prevent growth of the parental EL4 cells. Similar results were observed when ICD protein was used as the vaccine (data not

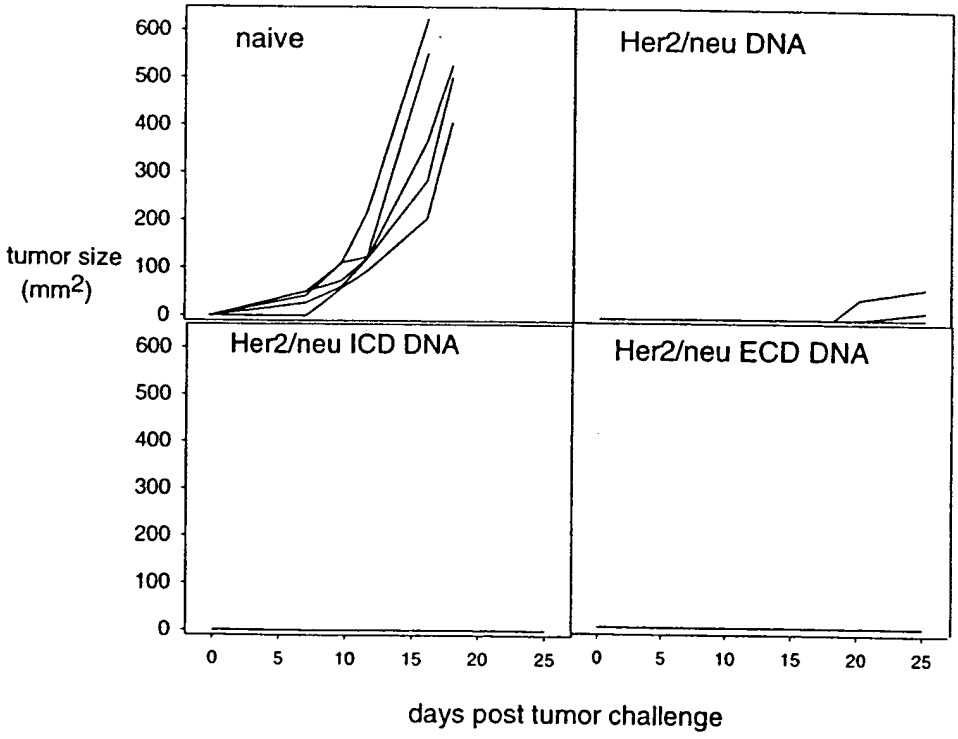

Fig. 1. Growth of EL4-Her-2/neu is inhibited by vaccination with plasmid DNA encoding Her-2/neu. Mice (five per group) were immunized (i.m.) with pVR1012-Her-2/neu, pVR1012-ECD or pVR1012-ICD (100 ug) on day 0 and 21. Mice were challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 35. Tumor size was monitored for 25 days following tumor challenge.

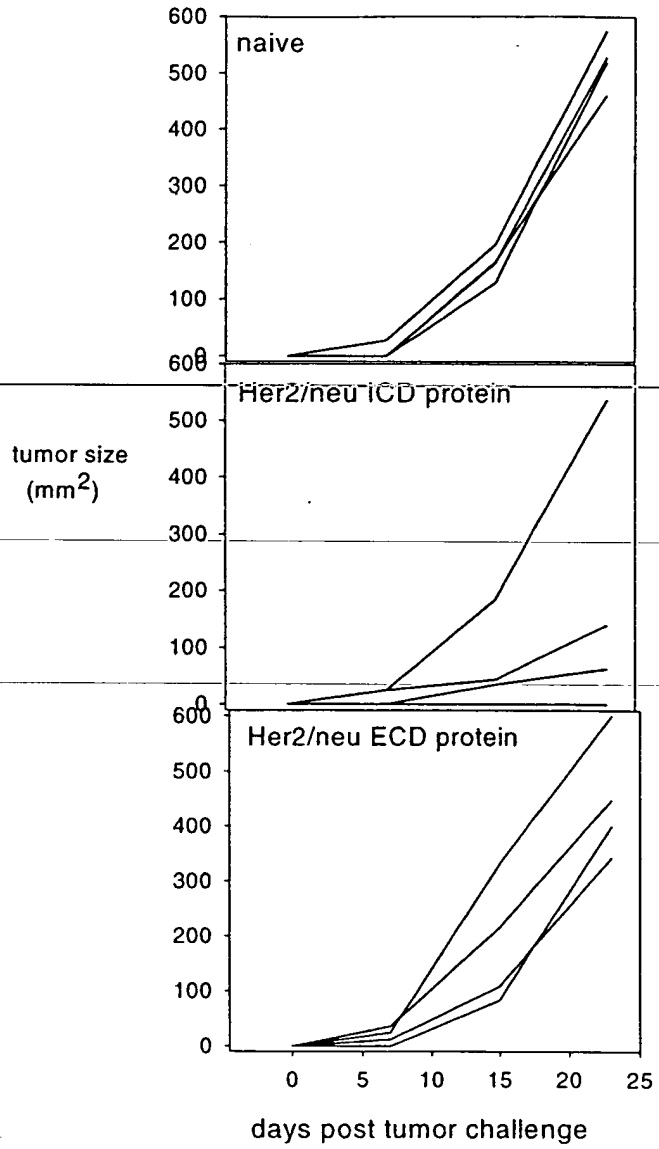

Fig. 2. Growth of EL4-Her-2/neu is partially inhibited by vaccination with the Her-2/neu ICD, but not ECD protein subunit. Mice (four per group) were immunized (s.q.) with Her-2/neu ICD or Her-2/neu ECD protein (50 ug) in Montanide 720 on day 0 and 21. Mice were challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 35. Tumor size was monitored for 25 days following tumor challenge.

shown). Taken together, these results indicate that tumor protection mediated by Her-2/neu vaccines is Her-2/neu specific.

3.2. Mechanism of tumor protection mediated by Her-2/neu protein subunits or plasmid DNA vaccines

In order to determine the nature of the immune response responsible for mediating tumor protection with Her-2/neu plasmid DNA or protein vaccination we next performed a series of in vivo depletion and adoptive transfer experiments. The first experiments were designed to evaluate the respective roles of CD4 and CD8 effector T cells. Mice were immunized twice with full length Her-2/neu or control plasmid DNA. Two weeks following the second immunization, mice were treated in vivo with *anti*-CD4 or *anti*-CD8 antibodies to deplete effector T cells. Greater than 98% CD4 or CD8 splenic T cell depletion was achieved by three administrations of antibody over the course of 7 days. Three days later, mice were challenged with EL4-Her-2/neu and monitored for tumor growth. Fig. 4 depicts the results of one representative experiment in which CD4 and CD8 effector T cells were depleted following Her-2/neu DNA vaccination. Consistent with the previous experiment shown in Fig. 1, complete tumor protection is observed in mice which were vaccinated with Her-2/neu plasmid DNA (untreated group). In contrast, in vivo depletion of CD4, but not CD8, effector T cells completely abrogates tumor protection mediated by Her-2/neu DNA vaccination. Similar results were obtained in adoptive transfer experiments where it was observed that adoptive transfer of CD8-, but not CD4-depleted effector T cells conferred protection against tumor challenge (data not shown). Collectively, these results suggest that in this tumor model, protection mediated by plasmid DNA vaccination is dependent upon the presence of CD4, but not CD8 effector T cells.

Similar experiments were carried out following vaccination with ICD protein to determine the roles of CD4 and CD8 T cells in the immune response elicited by this vaccine. Again, mice were immunized and boosted with ICD protein in adjuvant, depleted of CD4 and CD8 effector T cells by in vivo antibody treatment, and subsequently challenged with EL4-Her-2/neu. The results, shown in Fig. 5, indicate that depletion of either CD4 or CD8 T cells abrogates the partial protection obtained with ICD vaccination, suggesting that both CD4 and CD8 effector T cells play a role in ICD protein-mediated tumor protection. Results of adoptive transfer experiments also indicate that both CD4 and CD8 effector cells are important in the immune response elicited by ICD protein vaccination (data not shown).

Because it is known that *anti*-Her-2/neu antibodies can exhibit *anti*-proliferative effects on tumor cells, we investigated whether antibodies elicited by either plasmid DNA or protein vaccination contributed to the observed protection. In order to address this question, mice were immunized and boosted with full length Her-2/neu DNA or ICD protein. Sera from Her-2/neu immune mice or control sera from non-immune mice were collected and then transferred into naïve mice which were then challenged with EL4-Her-2/neu. The results from Her-2/neu DNA immune sera, shown in Fig. 6, indicate that transfer of antibody does not confer protection. These results are somewhat predictable given that the levels of *anti*-Her-2/neu antibod-

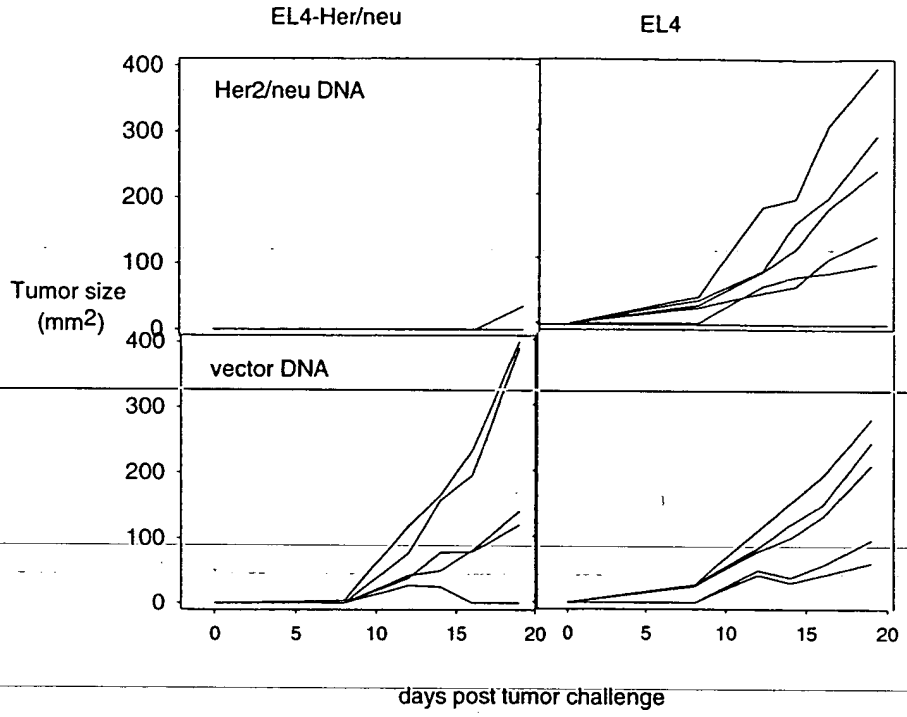

Fig. 3. Tumor protection is Her-2/neu specific. Mice (five per group) were immunized i.m. with pVR1012 or pVR1012-Her-2/neu (100 ug) on day 0 and 21. Mice were challenged with 200 000 parental EL4 cells or EL4-Her-2/neu cells subcutaneously on day 35. Tumor size was monitored for 25 days following tumor challenge.

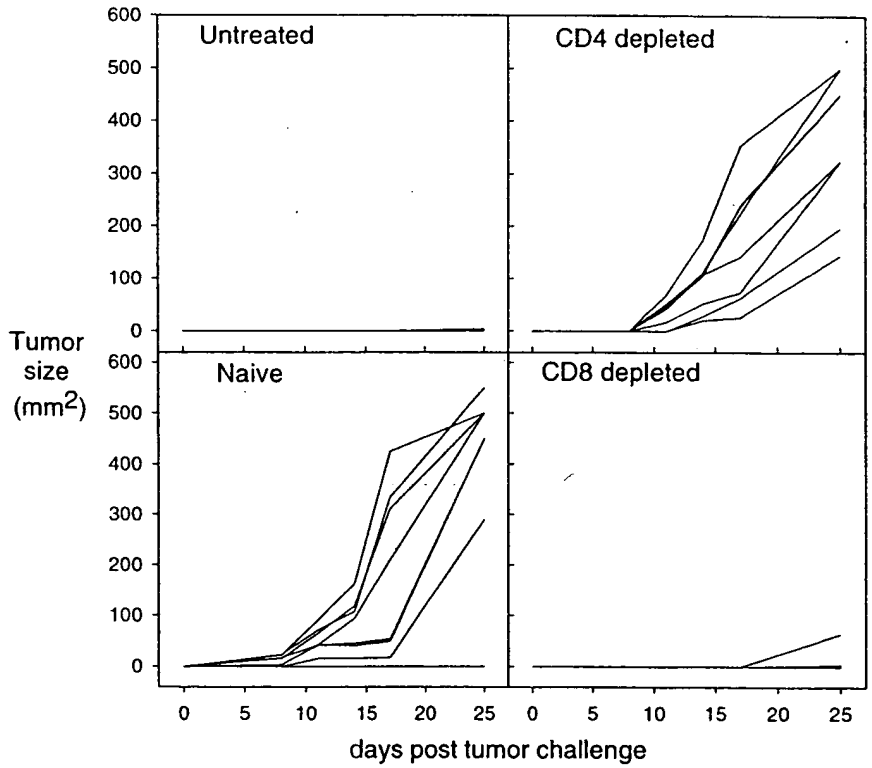

Fig. 4. Tumor protection mediated by Her-2/neu plasmid DNA vaccination requires CD4 T cells. Mice (eight per group) were immunized (i.m.) with pVR1012-Her-2/neu on day 0 and 21. On day 35, 38, and 42 mice were injected i.p. with 100 ug *anti*-CD4 or *anti*-CD8 antibodies to deplete effector T cells. Mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 45. Tumor size was monitored for 25 days following tumor challenge.

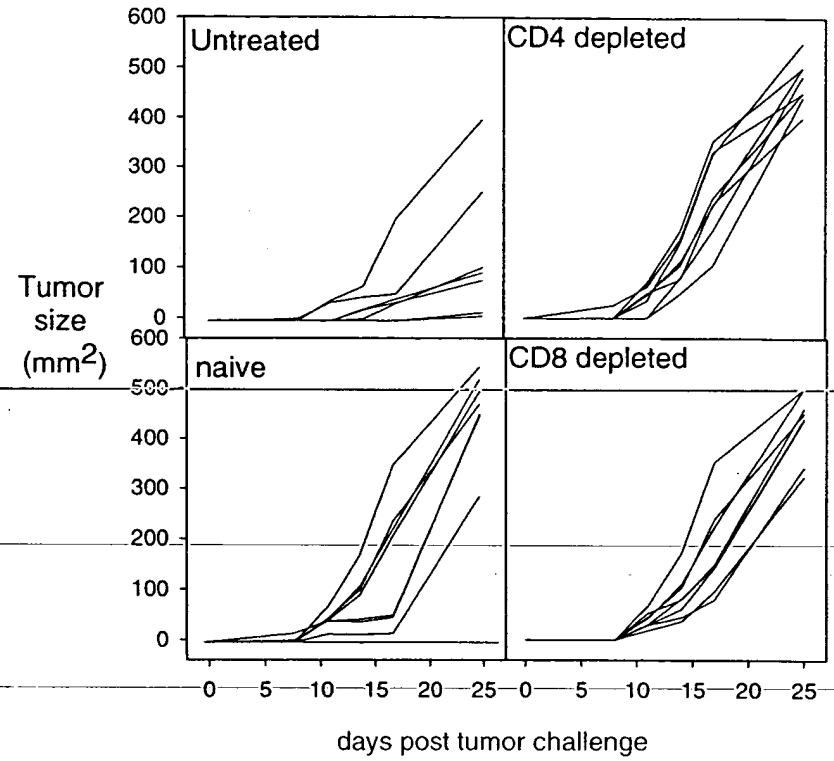

Fig. 5. Tumor protection mediated by Her-2/neu ICD protein subunit vaccination involves both CD4 T and CD8 T cells. Mice (eight per group) were immunized (s.q.) with Her-2/neu ICD protein (50 ug) in Montanide 720 on day 0 and 21. On day 35, 38, and 42 mice were injected i.p. with 100 ug *anti*-CD4 or *anti*-CD8 antibodies to deplete effector T cells. Mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 45. Tumor size was monitored for 25 days following tumor challenge.

ies obtained with plasmid DNA vaccination are quite low (data not shown). Similarly, transfer of *anti*-ICD containing sera was not protective (Fig. 7), despite the presence of substantial titers (10 000–100 000) of *anti*-ICD antibody present in this sera (data not shown). Taken together, these results suggest that antibody does not mediate the protection observed in this model using EL4-Her-2/neu tumor cells.

Results of these in vivo depletion and adoptive transfer experiments indicate that CD4+ T cells play a major role in the elicitation of a protective anti-tumor immune response in this model. In order to more fully elucidate the mechanism by which CD4+ T cells mediate protection, we examined the cytokine secretion profile of T cells following vaccination with either Her-2/neu DNA or ICD protein. The results, summarized in Table 1, demonstrate that upon in vitro restimulation with recombinant ICD or ECD protein, spleen cells from Her-2/neu plasmid DNA vaccinated mice secrete substantial levels of IFNγ compared to unstimulated cells. Spleen cells from ICD protein vaccinated mice also produced IFNγ in response to in vitro stimulation with ICD, but as expected, not ECD protein. The levels of IL4 and IL-5 in these same cultures were below detection, consistent with a Th1-type immune response. Taken together, these results suggest that IFNγ may play a role in the protection mediated by our Her-2/neu vaccines in this model.

4. Discussion

The present study demonstrates the utility of a Her-2/neu vaccine for the inhibition of tumor cell growth in vivo. We have shown that immunization with plasmid DNA encoding either full length or subunits of Her-2/neu effectively prevents growth of EL4-Her-2/neu in vivo in an antigen specific manner. Furthermore, our results indicate that protection mediated by Her-2/neu DNA is predominately CD4 and not CD8 or antibody dependent. Similar experiments carried out to evaluate Her-2/neu protein subunit vaccines indicate that ICD, but not ECD protein vaccination elicits partial tumor protection. In contrast to plasmid DNA-induced immunity, tumor protection observed with ICD protein vaccination involves both CD4 and CD8 effector T cells. Finally, analysis of the antigen-specific IFNγ production following protein and DNA immunization suggests that IFNγ is likely to play a role in elimination of Her-2/neu expressing tumor cells in vivo. Taken together, these data provide strong evidence to suggest

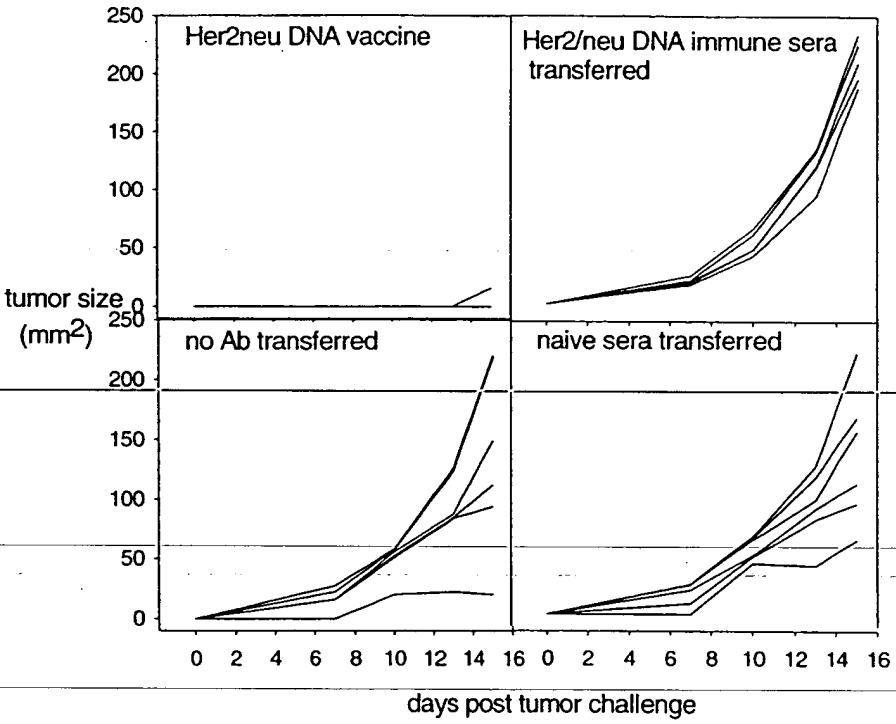

Fig. 6. Tumor protection mediated by Her-2/neu plasmid DNA vaccination is not antibody dependent. Mice (12 per group) were immunized (i.m.) with pVR1012-Her-2/neu on day 0 and 21. On day 35 immune sera were collected from the mice, pooled and transferred into six naïve recipient mice. Recipient mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously. Tumor size was monitored for 25 days following tumor challenge.

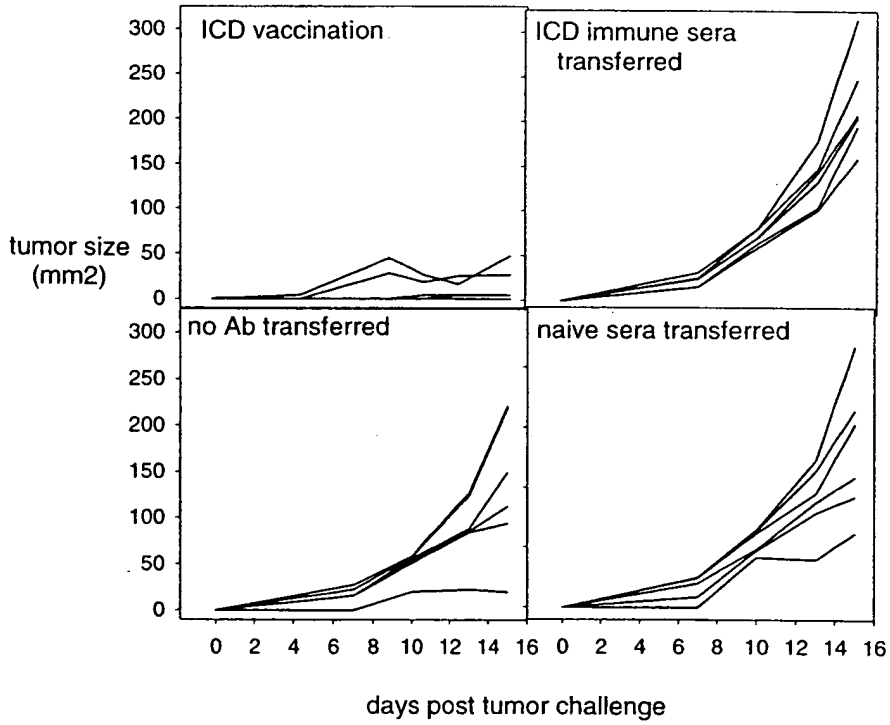

Fig. 7. Tumor protection mediated by Her-2/neu ICD protein subunit vaccination is not antibody dependent. Mice (12 per group) were immunized (s.q.) with Her-2/neu ICD protein (50 ug) in Montanide 720 on day 0 and 21. On day 35 immune sera were collected from the mice, pooled and transferred into six naïve recipient mice. Recipient mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously. Tumor size was monitored for 25 days following tumor challenge.

Table 1
IFNγ production following Her-2/neu DNA or protein vaccination

Vaccine[a]	IFNγ (ng/ml)		
	Medium[b]	ICD	ECD
pVR1012-Her-2/neu	0.32[c]	4.17	1.27
pVR1012	0.61	0.36	0.42
ICD protein	0.31	2.16	.01
Adjuvant alone	1.30	1.23	1.35

[a] Mice (four per group) were immunized with 100 ug pVR1012 or VR1012-Her-2/neu (i.m) or 50 ug of ICD protein in Montanide (s.q.), or Montanide alone on day 0 and 21.

[b] Two weeks following the second immunization, spleen cells were harvested and stimulated in vitro with media alone, ICD or ECD protein (10 ug/ml).

[c] IFNγ secretion was assayed by ELISA 48 h following in vitro stimulation. Values represent the mean of tripicate wells for four individual mice.

that protective immunity to Her-2/neu can be elicited by vaccination, demonstrating its utility as a cancer vaccine target.

In the present study, we chose to evaluate vaccines consisting of truncated forms or subunits of Her-2/neu because of the potential safety concerns regarding the use of a functionally active form of an oncogene in a vaccine. Her-2/neu, a known proto-oncogene has the potential to transform cells expressing it and therefore it may be difficult to gain approval for its use in humans. The results of plasmid DNA vaccine experiments demonstrate that tumor protection is effectively achieved using all three forms of Her-2/neu, supporting the contention that safety issues can be circumvented through the use of non-functional, truncated forms of the Her-2/neu antigen without compromising the quality of the protective response. These results are consistent with those obtained by other groups demonstrating protective immunity following plasmid DNA immunization with neu in FVB/N rat neu transgenic mice [13–15]. Our results also support those of Wei et al. who demonstrated protection using a mutated form of Her-2/neu in which the kinase activity had been eliminated [16]. More importantly, our data extend their observation to show that protective immunity can be achieved with smaller, truncated versions of Her-2/neu.

One primary aim of the current study was to determine the mechanism by which our Her-2/neu vaccines confer anti-tumor immunity. Numerous studies have established that both CD4+ and CD8+ T cells are required to achieve systemic anti-tumor immunity, whereas antibodies have been thought to play a lesser role. It has been proposed that CD8+ T cells are the predominant effector cells involved in anti-tumor immune responses (reviewed in [17]) and that CD4+ T cells are important for providing help to CD8+ T cells during priming [18–20]. There is a growing body of evidence, however, to suggest that CD4+ T cells can also play a direct role in mediating tumor immunity [21–24]. In the model presented in this study, T cells are clearly the major effector mechanism mediating tumor protection. This appears to be the case regardless of the composition of the Her-2/neu vaccine (i.e. plasmid DNA or protein). It is interesting to note, however, that the effector T cell population(s) responsible for protection mediated by DNA and protein are distinct. CD4+ T cells are the major effectors for plasmid DNA mediated protection, whereas both CD4+ and CD8+ T cells seem to be important in tumor protection following protein vaccination.

The observation that CD4+ T cells are the primary effector cells generated as a result of DNA vaccination is not without precedence. Although it is well documented that intramuscular plasmid DNA vaccines are very efficient at eliciting CTL responses (reviewed in [25]), evidence suggests that elicitation of CD4+ T-helper type 1 (Th1) T cell responses is also a property of DNA vaccines [26]. Moreover, there is increasing evidence to support the role of CD4+ T cells of both the Th1 and Th2 types in mediating anti-tumor immunity by indirect mechanisms. Mumberg et al. demonstrated that CD4+ T cells can eliminate MHC class II-negative tumor cells in vivo by an indirect IFNγ-dependent mechanism which likely stimulates other host cells to become tumorcidal [27]. Similarly, Hung et al. confirmed that cytokine production by CD4+ T cells plays an essential role in the recruitment of other anti-tumor effector cells such as macrophages and eosinophils in vivo [24]. Our observation that IFNγ secretion is readily detectable upon in vitro restimulation of immune spleen cells from Her-2/neu DNA-immunized mice suggests that CD4+ T cell secretion of IFNγ may likely be a component of the protective mechanism in this model. Current studies are underway to more specifically define the role of CD4+ T cells in this model.

Tumor protection resulting from protein immunization in this model appears to require both CD4+ and CD8+ T cells, indicating perhaps a more conventional role for CD4+ T cells in providing help to CD8+ T cells following protein vaccination. However, we were unable, despite exhaustive efforts, to detect Her-2/neu specific CTL following immunization in this model, suggesting that Her-2/neu specific CD8 T cells may be at a low precursor frequency, or alternatively, may be mediating protection via an effector mechanism other than direct anti-tumor cytolysis. Finally, although it is unclear why vaccination with the ICD subunit protein provides tumor protection when the ECD subunit protein does not, the observation that both proteins elicit detectable antibody responses supports the contention antibody does not mediate protection in this model.

The present study demonstrates the utility of multiple forms of Her-2/neu vaccines for the elicitation of protective Her-2/neu specific anti-tumor responses. Current studies are underway to comparatively evalaute these distinct formulations in primate models in an attempt to develop vaccines useful for treating human Her-2/neu expressing carcinomas.

References

[1] Coussens L, Yang-Feng TL, Liao Y-C et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene, Science 1985; 230–1132.

[2] Semba K, Kamata N, Toyoshima K, Yamamoto T. A v-*erb*B-related protooncogene, c-*erb*B-2, is distinct from the c-*erb*-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 1985;82:6497.

[3] Slamon DJ, Godolphin W, Jones LA, et al. Studies of the *Her-2/neu* proto-oncogene in human breast and ovarian cancer. Science 1989;244:707.

[4] Ishikawa T, Kobayashi M, Mai M, Suzuki T, Ooi A. Amplification of the c-*erb*B-2 (*Her-2/neu*) gene in gastric cancer cells. Amer J Pathol 1997;151:761.

[5] Disis ML, Calenoff E, Murphy AE, et al. Existent T-cell and antibody immunity to Her-2/neu protein in patients with breast cancer. Cancer Res 1994;54:16.

[6] Pupa SM, Menard S, Andreola S, Colnaghi MI. Anbtibody response against c-*erb*B-2 oncoprotein in breast carcinoma patients. Cancer Res 1993;53:5964.

[7] Ioannides CG, Whiteside TL. T cell recognition of human tumors: implications for molecular immunotherapy of cancer. Clin Immunol Immunopath 1993;66:91.

[8] Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA. High titer Her-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. J Clin Oncol 1997;15:3363.

[9] Tuttle TM, Anderson BW, Thompson WE, et al. Proliferative and cutokine responses to class II Her-2/neu-associated peptides in breast cancer patients. Clin Cancer Res 1998;4:2015.

[10] Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberline TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same Her-2/neu-derived peptide. Proc Natl Acad Sci USA 1995;92:432.

[11] Kono K, Rongcun Y, Charo J, et al. Identification of Her-2/neu-derived peptide epitopes recognized by gastric cancer-specific cytotoxic T lymphocytes. Int J Cancer 1998;78:202.

[12] Weiner LM. Monoclonal antibody therapy of cancer. Semin Oncol 1999;26:43.

[13] Chen Y, Hu D, Eling DJ, Robbins J, Kipps TJ. DNA vaccines encoding full-length or truncated Neu induce protective immunity against Neu-expressing mammary tumors. Cancer Res 1998;58:1965.

[14] Amici A, Venanzi FM, Concetti A. Genetic immunization against neu/erbB2 transgenic breast cancer. Cancer Immunol Immunother 1998;47:183.

[15] Esserman L.J, Lopez T, Montes R, Bald L.N, Fendly B.M, Campbell M.J Vaccination with extracellular domain of p185neu prevents mammary tumor development in neu transgenic mice. 1999; 47: 337.

[16] Wei WA, Shi WP, Galy A, et al. Protection against mammary tumor growth by vaccination with full-length modified human erbB-2 DNA. Int J Cancer 1999;81:748.

[17] Melief CJM. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. Adv Cancer Res 1992;58:143.

[18] Greenberg PD, Cheever MA, Fefer A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt1 + 2-lymphocytes. J Exp Med 1981;154:952.

[19] Keene J, Forman J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. J Exp Med 1982;155:768.

[20] Kast WM, Bronkhurst AM, de Waal LP, Melief CM. Cooperation between cytotoxic and helper T lymphocytes in protection against lethal sendai virus infection. J Exp Med 1986;164:723.

[21] Greenberg PD. Adoptive T cell therapy of tumors: mechanisms in the recognition and elimination of tumor cells. Adv Immunol 1991;49:281.

[22] Greenberg PD, Kern DE, Cheever MA. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1 + 2-T cells. Tumor eradication does not require participation of cytotoxic T cells. J Exp Med 1985;161:1122.

[23] Ossendorp F, Menged E, Camps M, Filius R, Melief CJM. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II-negative tumors. J Exp Med 1998;187:693.

[24] Hung K, Hayashi R, Laford-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4+ T cells in the anti-tumor immune response. J Exp Med 1998;188:2357.

[25] Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Ann Rev Immunol 1997;15:617.

[26] Raz E, Tighe H, Sato Y, et al. Preferrential induction of Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc Natl Acad Sci USA 1996;91:9519.

[27] Mumberg D, Monach PA, Walderling S, et al. CD4 + T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-γ. Proc Natl Acad Sci USA 1999;96:8633.